Europäisches Patentamt

European Patent Office

Office européen des brevets

Bescheinigung   Certificate   Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr.   Patent application No.   Demande de brevet n°

02015247.6

> # PRIORITY
> # DOCUMENT
> SUBMITTED OR TRANSMITTED IN
> COMPLIANCE WITH RULE 17.1(a) OR (b)

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

Europäisches
Patentamt

European
Patent e

Office européen
des brevets

Anmeldung Nr:
Application no.:   02015247.6
Demande no:

Anmeldetag:
Date of filing: 09.07.02
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

BASF Plant Science GmbH

67056 Ludwigshafen
ALLEMAGNE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Use of AHAS mutant genes as selection marker in patato transformation

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s)
revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C12N15/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of
filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR IE IT LI LU MC NL PT SE SK TR

Use of AHAS mutant genes as selection marker in potato trans-
formation

5 The present invention relates to an improved selection system,
using mutated acetohydroxy acid synthase enzyme (AHAS), for pro-
duction of transgenic potato lines. Genetic insertion of mutated
AHAS genes can give plants tolerance to a number of herbicide
compounds. This invention yields a higher transformation effi-
10 ciency and a reduced escape rate than previously described selec-
tion systems for patato plants.

The invention relates to a method for the generation of stably
transformed fertile plants of the genus *Solanum*, which comprises
15 the following steps:

(a)　growing the plant to be transformed, and obtaining the suit-
able explant,

20 (b)　transferring DNA sequences into plant cells,

(c)　selecting transformed plant cells and

(d)　regenerating transgenic fertile plants. Moreover, the method
25　　　comprises the possibility of transferring homologous or het-
erologous DNA sequences into the plant to be transformed or
explants thereof, for example by *Agrobacterium tumefaciens*.
Moreover, the method comprises the possibility of using
leaves as explants.
30

Acetohydroxyacid synthase (EC 4.1.3.18; AHAS; acetolactate sy-
thase) is an enzyme catalysing, in two parallel pathways, the
first step of the synthesis of the branched-chain aminoacids
valin, leucin and isoleucin. In the valin and leucin bio-
35 synthesis, AHAS is catalysing the production of acetolactate by
condensation of two pyruvate molecules. While in the isoleucin
biosynthesis AHAS condensates one pyruvate molecule with one
2-oxobutyrat molecule to form acetohydroxybutyrat (Umbarger,
1975). Sequence comparison of the AHAS gene in higher plants
40 shows high conservation in at least 10 regions. These regions are
probably of great importance for the AHAS function. In tobacco
two unlinked genes named SuRA and SuRB code for the AHAS enzyme
catalytic subunit. This is not the case in *Arabidopsis thaliana*
where only one gene is coding for the enzyme.
45

2

AHAS is the target enzyme of several classes of herbicides including sulphonylureas (Ray, 1984), imidazolinones (Shaner et al, 1984), triazolopyrimidines (Subrimanian et al, 1989) and pyrimidinyl oxybenzoat (Hawkes et al, 1989). The herbicides pre-

5 vent branched amino acids to be synthesised by the plant and may lead to plant death. However, mutations in the AHAS genes can result in higher tolerance to these herbicides (US 5,013,659) because of reduced affinity between enzyme and the herbicide.

10 In plants mutations conferring resistance to herbicides occurs as a result of exposure to the compound repeatedly as it was reported for *Arabidopsis thaliana*. Once the mutated genes are isolated they can be used to genetically engineer plants for improved tolerance to the herbicides. For example mutations

15 in the *Arabidopsis thaliana* AHAS gene have been produced and successfully confer resistance to imidazolinones as described in WO 00/26390, US 5,767,366 and US 6,225,105. Mutations in a corn AHAS gene confer imidazolinone resistance to monocot plants as described in EP 0 525 384.

20
The mutated AHAS genes can be used for production of herbicide resistant plants, yielding field resistance to a specific herbicide or can be used as a selection marker for genetic engineering of plants.

25
When genetic material is introduced into a population of plant cells, only a minor part of the cells are successfully transformed. For the production of novel genetically modified plants a selection system is transformed together with the trait genes

30 providing the transformed cells with a selective growth advantage. This construct allows to select transformed from non-transformed cells by adding a compound favoring the regeneration of transformed shoots.

35 A number of selection systems have been developed; most of them are based on the use of herbicide or antibiotic resistance genes. For many plant species, several of the used selectable marker genes yield a low transformation efficiency and many non-transgenic escapes. Thus more efficient selection systems are re-

40 quired.

The use of antibiotic and herbicide resistance is called negative selection due to that the non-transformed cells are greatly retarded in growth or even killed. Selectable marker genes which

45 can be used are for example the bialophos resistance gene (bar) and the kanamycin or G418 resistance gene (NPTII). By growing the tissue on media containing for example kanamycin, in theory only

3

the transformed cells will divide and take part in morphogenesis.
The NPTII gene encodes a neomycin phosphotransferase, which
reduces the inhibitory action of kanamycin, neomycin, G418 and
paromomycin by a phosphorylation reaction.

5

In contrast to negative selection research has also been done on
positive selection, based on giving the transformed cells a meta-
bolic advantage while the non-transformed cells are starving with
a concomitant slow reduction in viability. An example is the use

10 of the mannose or xylose based selection system as described
e.g. in US 5,767,378.

Solanum tuberosum is one of the major target crops for genetic
engineering. Main traits for potato are tuber quality, nutri-

15 tional composition, starch quality, starch yield, insect and
virus resistance. One trait with high market potential is the
production of high amylopectin starch. The high amylopectin
starch has an improved performance in the adhesive and paper
industry compared to ordinary starch. In the paper production it

20 will be used as a binder and for coating with printing quality
better than latex used today.

Solanum tuberosum is a tetraploid plant with a high level of
genetic heterozygosity. Conventional breeding of potato is there-

25 fore complicated because of segregation of important charac-
teristics. Genetic engineering has during the last decade been an
alternative to conventional breeding when it comes to improving
potato varieties. A single trait or a combination of traits are
more efficiently introduced by transformation than by using con-

30 ventional breeding. Due to the fact that genetically modified
potato plants can efficiently be vegetatively propagated the
trait is fixed in the commercial line without the normal subse-
quent breeding.

35 Agrobacterium tumefaciens mediated transformation has success-
fully been achieved in potato since 1986. Almost exclusively the
nptII gene has been used as a selection marker in potato.

The highest documented efficiency using nptII as selection marker

40 in potato transformation is described by Libiakova, G. et al.
in 2001. They have produced 120 leaf discs. Of those 81% produced
one or more shoots. 90% of the shoots were confirmed transfor-
mants and showed resistance to kanamycin. This is equivalent to a
regeneration efficiency of 81%, an escape rate of 10% and yields

45 a transformation efficiency of 73%.

4

It is an object of the present invention to provide an efficient
method for transformation of potato plants with a low escape rate
and a high transformation efficiency.

5 Leaf segments are the explants of choice used in Agrobacterium
mediated potato transformation. Transgenic plants are regenerated
via direct or indirect organogenesis under selective conditions.
Cytokinines as growth hormones are mainly involved in provoking
cell division and adventitious bud formation in in vitro potato
10 leaf segments. When Imazamox - (RS)-2-(4-isopropyl-4-methyl-
5-oxo-2-imidazolin-2-yl)-5-methoxymethylnicotinic acid, an
imidazolinone type herbicide - is applied in low concentrations
it was surprisingly found that it possesses a cytokinine-like
effect in tissue culture, increasing plant cell growth. At
15 higher concentrations the substance is acting as an herbicide.
The invention shows that Imazamox can efficiently be used as a
selective agent in the recovery of transgenic potato plants and
as a cytokinine-like substance it is supporting regeneration
during transformation. This phenomenon classifies Imazamox as
20 a unique selection agent that is combining both lethality for
non-transgenic cells and improved growth for transgenic cells.

The present invention is describing a new selection system for
efficient recovery of transgenic plants. The invention is also
25 describing an innovative selection system that is acting as
restriction for untransformed cells and support for transformed
ones. Finally the invention is presenting an efficient system for
recovery of transgenic potato plants and yields only a very small
escape rate.

30
The present invention relates to the use of a mutated AHAS gene
conferring imidazoline type herbicide resistance resulting in a
highly efficient selection system for production of transgenic
potato lines.

35
The invention provides a key advantage by minimizing the escapes
to almost zero of all shoots regenerated without interfering with
the high number of shoots regenerated per explant.

40 Another advantage is that the method does not comprise a gene
conferring resistance to an antibiotic.

For example a mutated gene coding for a functional AHAS enzyme
with tolerance to the imadazolinone type herbicides as described
45 in Sathasivan, K. et al, 1991 is used, see SEQ-ID No. 1. The
mutated AHAS gene is used for insertion as a selection marker
together with any other homologous or heterologous gene into

the potato genome for production of transgenic potato plants.
The AHAS enzyme naturally exists in higher plants and the use
of a modified plant AHAS gene as selection marker conferring
resistance to AHAS inhibiting herbicides does not result in the
5 addition of a new biosynthetic function.

Furthermore

(i)   the mutated AHAS gene can be of eukaryotic or prokaryotic
10   origin;
(ii)   the mutated AHAS gene can be regulated by any promoter
        functional in plants;
(iii)   any plant transformation method for insertion of the gene
        constructs can be used;
15 (iiii) any mutations in the AHAS gene resulting in tolerance to
        an AHAS   inhibiting herbicide can be used;
(iiiii) any potato variety can be transformed.

A mutated AHAS gene with mutation S653N as described in
20 SEQ-ID No. 1 and US 5,767,366 was cloned in a vector with the
reporter gene beta-glucuronidase (GUS) (Jefferson, R.A. et al.,
1987) and transformed to potato cells, see example 1 to 4.
The GUS activity is tested in the transgenic lines produced
and results in a transformation frequency of 93 to 100% of all
25 transgenic shoots analysed. The transformation method produces
a very high amount of shoots per explants, 3 to 5 independent
transgenic shoots dependent on the variety used. With a cal-
culation on transformation efficiency per explants the number
can be as high as 500%. This is the highest transformation fre-
30 quency of potato published. The mutated AHAS gene has also been
used in combination with a *gbss* antisense gene for production
of high amylopectin potato lines, see example 12. The AHAS
selection system yielded in this case the same high trans-
formation efficiency, which establishes the use of the AHAS gene
35 as a selection marker also for commercially important traits.

The highest efficiency of *nptII* as a selection marker is 74% (see
example 10) of totally analyzed shoots yielding a transformation
efficiency of 370% at the maximum. Another selection system that
40 might be used for potato transformation is the Mannose or xylose
based selection system. High percentage of shoots positively
transformed has been published for sugar beet and tobacco when
using a very high mannose concentration, however the high con-
centration of the selective agent inhibit the number of shoots
45 per explant distinctively (Joersbo et al., 1998). There are until

6

today no published data of the efficiency of the mannose and
xylose based selection system in potato.

The process furthermore comprises the possibility that compounds
5 with herbicidal activity inhibiting the AHAS synthase can be
employed for the selection of transgenic potato plants by using
different combinations of growth regulators as for example
auxins, cytokinines and/or auxin or cytokinines conjugates for
the regeneration into intact transgenic plants.
10

The method is suitable for transforming the species Solanum
tuberosum.

The invention also relates to transgenic fertile Solanum plants
15 themselves which have been generated by the method described and
to their transgenic seeds.

The method according to the invention allows for the first time
to transform plant cells of the genus Solanum with a transforma-
20 tion efficiency of more than 93% to be regenerated into
transgenic plants as shown in example 9.

The method for the generation of stably transformed Solanum
plants is composed of the following steps: (a) growing the plant
25 to be transformed and obtaining the suitable explant, (b) trans-
ferring DNA sequences into plant cells, (c) selecting transformed
plant cells with compounds which inhibit in non transformed plant
cells the AHAS-synthase and (d) regenerating these transformed
plant cells into fertile transgenic plants.
30

A variety of methods is currently available for the transforma-
tion. The most frequently employed method for transforming dicot-
yledonous plants is the Agrobacterium-tumefaciens-mediated gene
transfer. This method exploits the natural ability of the soil
35 bacterium to integrate genetic material into the plant genome.
Other suitable methods are for example protoplast transformation
by polyethylene-glycol-induced DNA uptake, electroporation, so-
nication or microinjection, the transformation of intact cells or
tissue by micro- or macroinjection into tissue or embryos, tissue
40 electroporation, incubation of dry embryos in DNA-containing
solution, vacuum infiltration of seeds and the biolistic gene
transfer.

The use of Agrobacterium tumefaciens for the transformation of
45 plants using tissue culture explants has been described by Horsch
et al., Science 228(1985), 1229-1231; Fraley et al., Proc. Natl.
Acad. Sci. USA 80(1983), 4803-4807 and Bevans et al., Nature

7

304(1983), 184-187. Many strains of *Agrobacterium tumefaciens*
are capable of transferring genetic material, into *Solanum*
species such as for example the strains EHA 101, EHA105, LBA4404
and C58C1 with various disarmed Ti-plasmids.

5

The agrobacterial strain employed for the transformation of pota-
toes contains in addition to its disarmed Ti-plasmid a binary
plasmid with the T-DNA to be transferred which contains a gene
for selecting the transformed cells and the gene to be trans-

10 ferred. Both genes must be equipped with transcriptional and
translational initiation and termination signals. The binary
plasmid can be transferred into the agrobacterial strain for
example by electroporation or other transformation methods, see
Mozo & Hooykaas, Plant Mol. Biol. 16(1991); 917-918. Coculture of

15 the plant explants with the agrobacterial strain takes two to
three days.

Foreign genes can be expressed in a constitutive, inducible (by
biotic and abiotic factors), tissue-specific or development-spe-

20 cific way. A relatively constitutive expression is achieved for
example in plants by the cauliflower mosaic virus 35S promoter.
This expression has been described by Shewmaker et al., Virology
140, 281-288 (1985) and Gardner et al., Plant Mol. Biol. 6,
221-228(1986).

25

Both the direct and the indirect gene transfer are suitable
transformation methods. The *Agrobacterium*-mediated transformation
using a wide range of starting explants such as for example coty-
ledons, leaves, hypocotyls, shoots, roots, callus, mature and im-

30 mature seeds or floral tissue can successfully be employed. The
gene transfer can be effected both by simple cocultivation/in-
cubation or wetting with the agrobacterial strain and by a sup-
porting vacuum infiltration of the explants with the bacterial
culture in question. The use of feeder cultures as aids may be

35 advantageous in the process. Each agrobacterial strain which con-
tains a Ti- or Ri-plasmid with the genetic information required
for the transfer is suitable as vector for the transformation.
Suitable agrobacterial strains are for example EHA101[pEHA101],
EHA105[pEHA105], LBA4404[pAL4404], C58C1[pMP90] and

40 C58C1[pGV2260]. Viral vectors also seem to be suitable for the
transformation of *Solanum*. Other methods for transferring genetic
material into *Solanum* are for example the polyethylene glycol
(PEG)-mediated protoplast transformation, the electroporation,
the sonication or microinjection and the transformation of intact

45 cells or tissue by micro- or macroinjection into tissue or
embryos, tissue electroporation, incubation of dry embryos in
DNA-containing solution, the vacuum infiltration of seeds, see

8

also Eds. Galun, E. and Breiman, A. In: Transgenic Plants, Imperial College Press, 1997. The use of the particle gun for bombarding a wide range of explants such as for example leaves and callus, is also possible.

5

All gene transfer vectors which contain the border sequences necessary for transferring the T-DNA (left and right border, LB and RB respectively), carry one or more selectable marker or reporter genes under the control of suitable promoters and
10 terminators and/or contain further useful genes or target genes, also under the control of suitable transcriptional and translational regulatory units, can be employed for the *Agrobacteria*-mediated transformation. The transfer of DNA sequences into the *Solanum* plant to be transformed or explants thereof is preferably
15 done by *Agrobacterium-tumefaciens*-mediated gene transfer.

The regeneration medium according to the invention furthermore comprises growth regulators such as auxins and/or auxin conjugates, and cytokinins and/or cytokinin conjugates. Suitable
20 auxins are both natural and synthetic auxins. The natural auxin is indoleacetic acid (IAA), examples of synthetic auxins are 3-indolebutyric acid (IBA), 1-naphthylacetic acid (NAA) and the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). Other herbicides which act as auxins are also feasible as growth regulators.
25 Auxin conjugates are compounds of auxin (IAA) with aspartic acid, glucose, myoinositol and others. In the case of the cytokinins, again, natural cytokinins such as, for example, zeatin and synthetic components, such as 6-benzylaminopurine (BAP) and 6-furfurylaminopurine (kinetin) may also be employed. Zeatin
30 riboside is frequently employed as cytokinin conjugate. The auxins and the cytokinins can be employed in each case as individual components, but also as auxin or cytokinin mixtures. The concentration of the individual growth regulators is 0.05 to 10 mg/l, preferably 0.1 to 5 mg/l.
35

The transformation method according to the invention can be applied, inter alia, to Solanaceae species as Solanum tuberosum (potato), Lycopersicon esculentum (tomato) and pepper.

40 The mutant AHAS genes of the present invention confer resistance to imidazolinone herbicides. Types of herbicides to which resistance is conferred are described for example in US Patent Nos.: 4,188,487; 4,201,565; 4,221,586; 4,297,128; 4,554,013; 4,608,079; 4,638,068; 4,747,301; 4,650,514; 4,698,092; 4,701,208; 4,709;036;
45 4,752;323; 4,772,311 and 4,798,619.

9

Mutant AHAS genes conferring resistance to AHAS inhibiting
herbicides are described in US 5,013,659, US 5,141,870 and
US 5,378,824.

5　Other mutant AHAS genes of the present invention could also
confer resistance to sulfonylurea herbicides. Types of mutants
which confer sulfonylurea resistance are described for example
in US Patent Nos.: US 5,853,973 and 5,928,937.

10　Mutant AHAS genes conferring resistance to imidazolinone type
herbicides are described in WO 00/26390 and US 5,767,366.

Furthermore Duggleby, R.G. and Pang, S.S. in Journal of Bio-
chemistry and Molecular Biology 33(1), 1-36 (2000) describe muta-
15　tions of the AHAS genes which could be used in the invention for
conferring herbicide resistance to transgenic potato plants.

In WO 00/26390 additional genomic and cDNA sequences coding for
an eukaryotic AHAS small subunit protein are disclosed. The DNA
20　sequences and vectors are used to transform plants to produce
transgenic plants which possess elevated levels of tolerance or
resistance to herbicides such as imidazolinones.

It will be understood by those working in the field that the
25　nucleic acid sequence depicted in SEQ-ID No. 1 is not the only
sequence which can be used to confer imidazolinone-specific
resistance. Also contemplated are those nucleic acid sequences
which encode an identical protein but which, because of the
degeneracy of the genetic code, possess a different nucleotide
30　sequence. The invention also encompasses genes encoding AHAS
sequences in which the above-mentioned mutation is present, but
which also encode one or more silent amino acid changes in posi-
tions of the molecule not relevant for resistance to herbicides
or to the catalytic function. Also contemplated are gene
35　sequences from other imidazolinone resistant monocot or dicot
plants which have a mutation in the corresponding region of the
sequence.

For example, alterations in the gene sequence which results in
40　the production of a chemically equivalent amino acid at a given
site are contemplated; thus, a codon for the amino acid alanine,
a hydrophobic amino acid, can readily be substituted by a codon
encoding another hydrophobic residue, such as glycine, or may
be substituted with a more hydrophobic residue such as valine,
45　leucine or isoleucine. Similarly, changes which result in a
substitution of our negatively charged residue for another, such
as aspartic acid for glutamic acid, or one positively charged

10

residue for another, such as lycine for arginine, can also be
expected to produced a biologically equivalent product.

The invention also encompasses chimaeric genes, in which the
5 substituted portion of the corn or Arabidopsis AHAS gene is
recombined with unaltered portions of the normal AHAS gene, in
which the substituted portion of the corn or Arabidopsis AHAS
gene is recombined with unaltered portions of the normal AHAS
gene from other species. Thus, throughout the specification and
10 claims, wherever the term "herbicide resistant AHAS gene" is
used, it is intended to cover each of these alternate embodiments
as well as the sequence of SEQ-ID No. 1.

For expression of the mutated AHAS gene conferring herbicide
15 resistance in potato the following promoters can be used:

the tuber specific gbss-promoter from potato described in
WO 92/11376, and the patatin promoter described in Rocha-Sosa
et al., 1989 EMBO J. 8:23-29;
20
the light inducible promoter: cytosolic FBPase from potato
described in WO 98/18940;

the octopine promoters (US 5,428,147); the triple OCS enhanced
25 vATPase cl promoter from Beta vulgaris (Plant Mol Biol (1999) 39:
463-475);

Constitutive promoters: for reference see Benfey et al., EMBO
J. 8 (1989), 2195-2202; the 35S promoter (Franck et al., Cell 21,
30 (1980), 285-294) or enhanced versions; the 19S promoter, see
US 5,352,605 and WO 84/02913;

the RUBISCO small subunit SSU promoter: see US 4,962,028;

35
plastid specific promoter: see e.g. the RNA polymerase promoter
in WO 95/16783 and WO 97/06250 or the clpP-promoter in
WO 99/46394;

40 the AHAS promoter as described in US 6,025,541;

Other promoters for the expression of genes in the leaf, in the
callus, in specific tissues as e.g. in the tubers or other parts
of the potato plant could also be used.
45

11

The invention can especially be carried out by using the AHAS
promoter, the AHAS resistance gene S653N as described in
US 5,767,366 and the AHAS terminator from Arabidopsis thaliana
cut from pAC321 as described in example 3.

5

The Arabidopsis AHAS gene (S653N) used for transformation and
selection contains most of the common restriction sites for clon-
ing such as HindIII, BamHI, EcoRI, SstI, BglII and EcoRV. Alter-
ation of the molecular composition of the AHAS gene can be per-
10 formed to eliminate restriction sites without altering the amino
acid sequence of the resulting protein. Special care must be
taken not to alter the codon usage profile from the one found in
Arabidopsis, which could lead to reduced translation efficiency.

15 The designed gene can be made synthetically and accommodates
suitable restriction sites at 5' and 3' ends for cloning.

Comparative studies have to be performed in potato in order to
confirm that the synthetically made gene does not differ from the
20 original mutated AHAS gene in conferring herbicide resistance.

For selection of transgenic potato plants chemical compounds in-
hibiting the AHAS enzyme can be used. Usefull compounds are the
imidazoline type herbicides. Especially useful compounds are se-
25 lected from the group consisting of imazethapyr (Pursuit™), ima-
zamox (Raptor™), imazamethabenz (Assert™), imazapyr (Aresenal™),
imazapic (Cadre™) and imazaquinon (Scepter™).

For selection of transgenic plants chemical compounds as
30 described in the review article by Duggleby, R.G. and Pang, S.S.
in Journal of Biochemistry and Molecular Biology 33(1), 1–36
(2000) can be used.

The mutated AHAS gene as described in SEQ-ID No. 1 has been
35 established as a selection marker in five different potato
varieties, see table 2 and 3, and can thus be used for trans-
formation of potato varieties in general. Two different selection
pressures, using 0.3 and 0.5 µM Imazamox, have been tested,
see table 2. For selection on 0.3 µM Imazamox the transformation
40 efficiency is ranging from 7 to 76 % depending on the variety
used. When increasing the selection to 0.5 µM Imazamox the trans-
formation rate is increasing to 93 to 100% respectively. The high
transformation efficiency at 0.5 µM Imazamox is independent from
the variety used. The fifth variety Seresta has as can be seen in
45 table 3 and 4 a transformation efficiency of 98 to 100% at 0.5 µM
Imazamox.

12

The invention furthermore relates to a plant expression vector
comprising SEQ-ID No. 1 wherein the heterologeous DNA sequence
encodes a peptide, protein, antisense-, sense-RNA, viral RNA or
ribozyme.

5

The invention furthermore relates to a plant expression vector
comprising SEQ-ID No. 1 wherein the heterologeous DNA sequence
contains information that causes changes in the carbohydrate
concentration and the carbohydrate composition of regenerated
10 potato plants.

The invention furthermore relates to a plant expression vector
comprising SEQ-ID No. 1 wherein the heterologeous DNA sequence
contains information that causes the increased production of
15 amylose type starches by using gene constructs as described in
WO 92/11375, WO 92/14827, WO 96/34968 and WO 97/20040.

The invention furthermore relates to the use of a DNA sequence
SEQ-ID No. 1 or a DNA sequence comprising a nucleotide sequence
20 which hybridizes to a complementary strand of the nucleotide SEQ-
ID No. 1 or a DNA sequence comprising a nucleotide sequence which
is degenerated to the nucleotide sequence SEQ-ID No. 1 or a DNA
sequence being a derivative, analogue or fragment of a nucleotide
sequence encoding a protein possessing AHA synthase activity and
25 conferring resistance to AHA synthase inhibitors.

The experiment shown in example 12 with the high amylopectin
trait establishes the use of the AHAS resistance gene conferring
resistance to herbicides inhibiting the AHAS enzyme as a selec-
30 tion marker also for commercially important traits. Trans-
formation efficiency is 98 and 100% respectively. Analysis of
the amylopectin/amylose ratio shows that in the transgenic shoots
selected the amylopectin content is higher than in non transgenic
control shoots. This is proof that by using the invention as
35 described commercially relevant transgenic potato plants can be
generated.

The invention now having been generally described will be more
readily understood by reference to the following examples, which
40 are included for the purpose of illustration only, and are not
intended to limit scope of the present invention.

45

13

Example 1

Test for transgenicity – Detection of glucuronidase expression

5 Microtubers of the regenerated in vitro potato plants were sub-
jected to a qualitative glucuronidase (GUS) enzyme detection by
infiltrating cut microtubers for 1 minute in vacuo with the GUS
substrate 5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid (X-GlcA)
in a 100 mM sodium phosphate buffer, pH 7.0, which contained
10 10 mM EDTA, 0.1% Triton X100 and 10 mM DTT, and subsequently
incubating them for approximately 15 hours at 37°C. Microtubers
showed an intense blue coloration, which proves that the reporter
gene is expressed in Solanum.

15 Example 2

Investigation of selection pressure

Potato leaf segments from in vitro propagated plants of four
20 different potato varieties Prevalent, Producent, Kuras and
Desiree were tested for natural tolerance on different concen-
trations of the imidazolinone herbicide Imazamox. It is important
to choose a selection concentration at which the potato leaf
tissue is viable long enough to regenerate shoots but have a high
25 enough concentration to prevent the regeneration of untransformed
shoots. Doses 0.1, 0.5, 1, 5, 10, 20, 30 and 50 μM Imazamox were
tested.

Fully expended potato leaves are diagonally cut in 2 pieces
30 and precultivated on MC-plates for 2 to 3 days at 23 to 24°C,
see table 1.

The leaf tissues are transferred on MS300 medium for additional
2 days and cultivated under modest light at 23 to 24°C simulating
35 a co-cultivation step. Subsequently the leaf segments are moved
to MS400 plates containing 400mg/l Claforan. Claforan is added
in order to suppress growth of Agrobacterium tumefaciens. There-
fore the regeneration of shoots on different concentrations of
Imazamox need to be monitored in presence of Claforan. Selection
40 is taking place within 4 to 5 days when the explants are moved to
MS400 medium supplemented with 400mg/l Claforan and Imazamox at
above concentrations.

The explants are transferred to fresh selection medium every
45 fortnight.

14

MC plates	MS300
MS300 plates with 1.5–2 ml liquid MS100 medium and covered with one sterile filter paper	X MS–medium 2mg/l NAA (naphtyl acetic acid) 1mg/l BAP (6–benzyl amino pyridine) 3% (w/v) sucrose pH 5.2
MS 10	MS400
4.4 g/l MS–medium (Murashige and Skoog) 1% (w/v) sucrose pH 5. 8	4.4 g/l MS–medium 2mg/l zeatine 0.01mg/l NAA (naphtyl acetic acid) 0.1 mg/l GA3 (giberellic acid) 10% (w/v) sucrose 400 mg/l claforan Imazamox or kanamycin pH5.8
MS 30	Microtuber medium
4.4 g/l MS–medium 3% (w/v) sucrose pH 5.8	4.4 g/l MS–medium 2.5 mg/l kinetin 0.5 mg/l ABA (abscisic acid) 8% sucrose 200 mg/l claforan
MS100	
4.4 mg/l MS–medium 30g/l sucrose 0.5 mg/l thiamin–HCl 0.5 mg/l pyridoxin–HCl 1 mg/l nicotinacid 0.5 mg/l kinetin 29.8 mg/l ferrous sulfate hepta hydrate 1 mg/l 2,4–Dichlorophenoxyacetic acid 2 g/l caseinhydrolysate pH 5.2	

Table 1: Media used for potato transformation.

Example 3

Construction of binary vectors pAHASGUS, pAP1 and pAP2

For all constructs the same AHAS mutant gene with mutation S653N originating from *Arabidopsis thaliana* was used (Sathasivan, K. et al., 1991).

pAHASGUS

AHAS gene with mutation S653N as described in SEQ ID No. 1, originating from *Arabidopsis thaliana*, was used together with the nos promoter (Herrera, L. et al., 1983) and the OCS terminator (Wesley S. V. et al., 2001) in the binary vector pGPTVkan (Becker, D. et al., 1992). The pBIN19 based pGPTVkan was

15

digested with ApaI and BamHI discarding the nptII gene. The
remaining 12600 bp contained from right boarder the nos termi-
nator, the uidA gene (also known as GUS), the cloning cassette,
the nos promoter and the pAg7 terminator. The AHAS gene with
5 mutation S653N and the OCS terminator was ligated to the digested
pGPTVkan and named pABA1, see figure 1.

For expression of the GUS gene a tuber specific gbss promoter
988bp (WO 92/11376) was cloned at the SmaI site of pABA1.
10

The resulting pAHASGUS construct was used for *Agrobacterium tume-
faciens* transformation of potato, where regenerated plantlets
were analysed for GUS expression to determine the transformation
efficiency.
15

pAP1

AHAS gene with mutation S653N, driven by the *Arabidopsis thaliana*
AHAS promoter as described in US 5,750,866, was used as a selec-
20 tion gene for co-transformation with a granule-bound starch syn-
thase (gbss) antisense gene (EP-A 0 563 189).

A 293 bp nos terminator was cut out from pHAxwO and ligated to
pBluescript with EcoRI. The 2952 bp gbss promotor and gbss gene
25 in antisense direction (EP-A 0 563 189) was cut from pHAxwO and
ligated in front of the nos terminator with HindIII resulting in
pMJ2. The gbss complex was cut out from pMJ2 with XbaI-XhoI and
ligated to pSUN1 (WO 02/00900) cut with XbaI-SalI resulting in
the construct named pMJ3.
30

pAC321 was cut with XbaI which yielded a fragment of 5717 bp con-
taining AHAS promoter, AHAS gene and AHAS terminator all origi-
nating from *Arabidopsis thaliana* as described in SEQ-ID No. 1.

35 SEQ-ID No.1 contains the following elements:

        1-2483 At AHAS promoter
        2484-4496 At AHAS gene
        4497-5717 At AHAS terminator
40

The pAC321 fragment was ligated to pMJ3 opened with XbaI.

The resulting pAP1 (figure 1) construct was used for *Agrobacter-
ium tumefaciens* transformation of potato, which were analysed for
45 high amylopectin starch quality.

16

pAP2

AHAS gene with mutation S653N, driven by the nos promoter
(US 6,174,724), was used as a selection marker for co-transforma-
5 tion with a gbss antisense gene (WO 92/11376).

A nos promotor of 600 bp was cut out from pGPTVKan (Becker et
al., 1992) with HindIII, blunted and cut with BglII. The fragment
was ligated to pBluescript (Stratagene) cut with SpeI, blunted
10 and cut with BamHI. The construct was named nPnos2.

A 2986 bp fragment of pACGH101 containing the AHAS gene with
mutation S653N as described in SEQ ID No. 1 and OCS terminator
was cut out with PstI and PvuII. The AHAS gene originates from
15 Arabidopsis thaliana see pAP1. The pACGH101 fragment was ligated
to pPnos2 cut with PstI and EcoRV and named pPnoas.

The 3406 bp AHAS complex was cut out from pPnoas with XbaI-PvuII
and ligated to pMJ3 (see pAP1) cut open with XbaI.
20
The resulting pAP2 (figure 2) construct was used for Agrobacter-
ium tumefaciens transformation of potato, which were analysed for
high amylopectin starch quality.

25 Example 4

Transformation method

Fully expanded leaves from in vitro propagated potato plants
30 are diagonally cut in 2 pieces and precultivated on MC-plates
for 2 to 3 days at 23 to 24°C.

Agrobacterium tumefaciens containing pAHASGUS is grown in YEB
medium containing 25 µg/ml kanamycin and 100 µg rifampicin and
35 grown over night on constant shaking (200 rpm) at 28°C.

The Agrobacterium culture is prepared for infection by dilution
1:20 with MS10 medium. The leaf explants are infected for
8-10 min in the bacterial solution and afterwards drained on
40 filter paper for 5 to 20 seconds. The leaf segments are placed
on the MS300 plates for 2 days co-cultivation under modest light
at 23 to 24°C. At the end of co-cultivation the leaf segments are
moved to M400 plates containing 400 mg/l Claforan to suppress
bacterial growth. After 4 to 5 days the explants are moved to
45 selection medium MS400 supplemented with 400 mg/l Claforan and
Imazamox at the previously detected concentrations of 0.3 or
0.5 µM.

17

Leaf segments are transferred to fresh MS 400 selection medium
every fortnight. The regenerated putative transgenic shoots are
collected and cultivated on MS30 plates with 200mg/l Claforan
aiming at shoot elongation.

5

When the shoots are 3 to 5 cm long, 1 to 2 cm are cut off and
grown on microtuber medium in the dark at 25°C. After 2 to 5 weeks
microtubers are produced. Putative transgenic plants are analysed
for GUS expression in microtubers to determine the transformation
10 efficiency.

Example 5

GUS expression analysis for determination of the transformation
15 efficiency

A thin slice from each microtuber was placed in a microtiter
plate. The slices were incubated with 1 mM X-gluc in 50 mM NaH_2PO_4
pH 7.2 at 37°C for approximately 1 hour. A very distinct blue
20 colour appeared on tuber slices if a successful integration and
expression of the GUS gene was achieved.

Example 6

25 Transformation efficiency determination of pAP1 and pAP2 by PCR
analysis

Transgenic shoots were distinguished from non-transgenic shoots
- for example escapes - by using PCR. DNA was extracted according
30 to DNeasy 96 Plant protocol (Qiagen, Germany). In a 96 well
microtiter plate, 10 to 15 mg leaf tissue was added to each well
together with a 5 mm steel ball each well then representing one
individual shoot. The plates were frozen in $N_2(l)$ before homo-
genisation. The homogenisation was done at 30 Hz in a Mixer-
35 mill 300 for 1 min. The DNA was at the end of the extraction
protocol eluted in 75 ml H_2O.

Specific primers were used to amplify a fragment of the mutant
S653N AHAS gene. Successful integration of the mutant AHAS gene
40 results in a fragment of 509 bp upon PCR amplification using the
specific primers.

Forward primer: AHAS1_frw: AACAACAACATCTTCTTCGATC
Reverse primer: AHAS1_rev: TAACGAGATTTGTAGCTCCG
45

18

The PCR reactions were done with the extracted DNA setup and run
as follows:

Reaction:

5

10x PCR Mix	2,0 µl
Primer frw (25 µM)	0,4 µl
Primer rev (25 µM)	0,4 µl
dNTPs (10 mM)	0,4 µl
10 RedTAQ (Sigma)	1,0 µl
Templat (~20 ng/µl)	4,0 µl
H_2O	11,8 µl

PCR program:

15

94°C	30 s
59°C	30 s x 29 cycles
72°C	30 s
72°C	7 min
20 8°C	Hold

Negative and positive control was included in all runs.
The reactions were analysed on 1.5% agarose gels.

25 Example 7

Starch quality analysis of amylopectin/amylose ratio

The screening for an altered amylopectin/amylose ratio was per-
30 formed on transgenic lines selected for high quality integration
of the intended genetic insert. The screening was done by stain-
ing with iodine (Lugol's solution: (6.7 g/l KI + 3.3 g/l I_2) and
(glycerol); ratio 1:1). Iodine stains starch containing amylose
blue and starch exclusively containing amylopectin red-brown. A
35 microtuber was crushed and a few drops of Lugol's solution were
added. The starch amylopectin quality was analysed under the
microscope according to the staining colour of the starch.

Example 8
40
Leaf tissues were tested for the use of the AHAS resistance gene
contained in SEQ ID No. 1 and for the selection of transgenic
potato plant cells as described above. Four different potato cul-
tivars Prevalent, Producent, Kuras and Desiree were cultivated
45 on different concentrations of Imazamox, an imidazolinones type
herbicides. It is essential to use the proper concentration for
selection to yield an acceptable number of shoots per explant

19

without high escape rate. The lethality of various Imazamox
concentrations on regeneration was evaluated.

Lethal dose for regeneration was detected within 0.1 to 1 µM
5 ranges of concentrations. Slight variation in lethality was
detected between varieties. No regeneration occurs when 0.5 µM
Imazamox was included in the medium. The concentrations 0.3 µM
and 0.5 µM are selected for transformation experiments providing
a lethality of 80 and 100% in regeneration.
10

Example 9

Transformation of four different potato varieties (Prevalent,
Producent, Kuras and Desirae) was performed with pAHASGUS as
15 described above. For selection a concentration of 0.3 or 0.5 µM
Imazamox was used. Each shoot was analysed according to GUS
activity and the number of GUS positive shoots in total is listed
in table 2. A distinct blue colour appears on the microtuber
slices if a successful integration of the GUS gene was achieved.
20 From each explant 3 to 5 independent shoots were regenerated
depending on the variety used. In all experiments the efficiency
of positive shoots per explants is stated and the figures
in table 2 have to be multiplied 3 to 5 times, yielding a trans-
formation efficiency of up to 500%. Compared with selection
25 systems used previously in potato plants the big advantage of the
invention described is that together with a very high efficiency
the number of shoots per explant is very high. For all four
varieties transformed an extremely high transformation efficiency
of up to 93% to 100% of all shoots regenerated was achieved at
30 an Imazamox concentration of 0.5 µM. The experiment using 0.3 µM
of Imazamox for selection shows how important the Imazamox
concentration is for an efficient transformation rate. This
can be seen from the results for variety Kuras in which a trans-
formation efficiency of 7% at 0.3 µM Imazamox is increased to 93%
35 at the 0.5 µM level.

40

45

20

Variety	Construct	Selection Imazamox (µM)	Selection Imazamox (mg/l)	Number analysed	Number of positive	% positive
Desiree	pAHASGUS	0.3 µM	0.0915mg/l	19	12	63
Prevalent	pAHASGUS	0.3 µM	0.0915mg/l	26	16	62
Producent	pAHASGUS	0.3 µM	0.0915mg/l	25	19	76
Kuras	pAHASGUS	0.3 µM	0.0915mg/l	58	4	7
Prevalent	pAHASGUS	0.5 µM	0.1525mg/l	23	23	100
Producent	pAHASGUS	0.5 µM	0.1525mg/l	40	38	95
Kuras	pAHASGUS	0.5 µM	0.1525mg/l	33	32	97
Desiree	pAHASGUS	0.5 µM	0.1525mg/l	29	27	93

Table 2: Transformation efficiency of four different varieties with 0.3 and 0.5 µM Imazamox used as the selective agent.

Example 10

In a similar experiment compared to example 9 the *nptII* gene was used as selection marker being controlled by the same promoter nos as in pAHASGUS. The shoots are selected on 50 µM kanamycin, which is a standard kanamycin concentration used for potato transformation as described in Ooms, G. et al., 1987 and Tavazza, R. et al., 1988. The transformation frequency was much lower compared to when using a mutated AHAS gene e.g. the S653N gene as selection marker. The highest transformation efficiency based on analysis of the same GUS gene driven by the same GBSS promoter resulted in 74% transformed shoots regenerated.

Example 11

The successful use of a mutated AHAS gene as a selection marker has been demonstrated using the promoter from the wild-type *Arabidopsis thaliana* AHAS gene (pAP1 construct) as well as a recombinant nos promoter (pAP2 construct); see table 3. This shows that any promoter with sufficient expression can be used in connection with the mutated AHAS gene and thus the invention could be used with different promoters as regulatory elements for the mutated AHAS gene successfully yielding selection of transgenic shoots on selective medium. The AHAS gene can be driven by different promoters such as e.g. the AHAS-, nos-, 35S-, Rubisco- or vATPase - promoter.

21

Variety	Construct	Promoter	Number shoots analysed	Number shoots positive	% positive
Seresta	pAP1	A.t AHAS	50	49	98
Seresta	pAP2	nos	120	117	98

Table 3: Transformation efficiency of the mutated AHAS gene driven
by two different promoters: *Arabidopsis thaliana* (A.t.)
AHAS promoter and the nos promoter.

Example 12

The mutated AHAS gene conferring resistance to Imazamox was used
as a selection marker for the transformation of a commercially
important trait to *Solanum tuberosum* resulting in potato lines
producing amylopectine type starch. The starch component amylose
is synthesized by granule-bound starch synthase (GBSS). Inhibi-
tion of the gene coding for GBSS directs starch production com-
pletely to amylopectin. An antisense gene fragment inhibiting the
expression of gbss as described in EP-A 0 563 189 was co-trans-
formed with the mutated AHAS gene conferring resistance to Imaza-
mox.

The gbss promoter was used to drive the gbss antisense gene
fragment. The Nos promoter or the AHAS promoter was used to drive
the AHAS selection gene and resulted in a total transformation
efficiency per shoots analysed of 98 to 100%, see table 4.

For restriction map of the T-DNA of the pAP1 and pAP2 constructs
used, see figure 4 and 5. There were 3 to 5 independent shoots
produced per explant yielding a transformation efficiency of 280
to 480% or up to 500% per explant. There were no differences in
transformation efficiency between the different promoters and
Solanum tuberosum varieties used.

22

Experiment	Variety	Construct	Number shoots analysed	Number shoots positive	% positive	high amylo-pectin content
1	Seresta	pAP2	120	117	98	+
2	Seresta	pAP1	50	49	98	+
3	Seresta	pAP2	30	30	100	+
4	Seresta	pAP2	3	3	100	+

Table 4: pAP1 and pAP2 transgenic lines analysed for trans-
formation efficiency and high amylopectin quality.

Starch quality analysis was done with microtubers of the
positive shoots with high quality integration of the intended
genetic insert. The starch was mixed with iodine and the shoots
exclusively containing amylopectin stains red-brown compared to
the starch containing amylose that stains blue.

23

References

Hawkes, T.R., in Prospects for Amino Acid Biosynthesis Inhibitors in crop protection and Pharmaceutical Chemistry, pp131-138,
5 Brittish Crop Protection Council Monograph No 42, Surrey, U.K. (1989)

Herrera-Estrella, L., Depicker, a., Van Montagu, M., Schell, J., Expression of chimaeric genes transferred into plant cells using
10 Ti-plasmid-derived vector, Nature 303:209-213 (1983)

Becker, D., Kemper, E., Schell, J., Masterson, R., New plant binary vectors with selectable markers located proximately to the left T-DNA border, Plant Molecular Biology 20:1195-1197, (1992)
15
Jefferson, R.A., Kavanagh, T.A., Bevan, M.W., GUS fusions: b-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, EMBO Journal 6: 3901-3907 (1987)

20 Joersbo, M., Donaldson, I., Kreiberg, J., Guldager Petersen, S., Brunstedt, J., and Okkels, F.T., Analysis of mannose selection used for transformation of sugar beet, Molecular Breeding 4:111-117 (1998)

25 Libiakova, G., Jorgensen, B., Palmgren, G., Ulskov, P., Johansen, E., Efficacy of an intron containing kanamycin resistance gene as a selectable marker in plant transformation, Plant Cell Rep 20:610-615(2001)

30 Ooms, G., Burell, M.M., Karp, A., Bevan, M., Hille, J., Genetic transformation in two potato cultivars with T-DNA from disarmed Agrobacterium,
Theoretical and Applied Genetics (1987) 73:744-750,

35 Ray, T.B., Plant Physiology (1984), 75, 827-831

Sathasivan, K., Haughn, G.W., Murai, N., Plant Physiology 97(1991), 1044-1050

40 Shaner, D.L., Andersson, P.C., Stidham, M.A., Plant Physiology 76 (1984), 545-546

Subrimanian, M.V., Gerwick, B.C., in Biocatalysis in Agricultural Biotechnology, pp277-288, ACS Symposium Series No389, American
45 Chemical Society, Washington DC (1989)

24

Tavazza, R., Tavazza, M., Ordas, R.J., Ancora, G., Benvenuto, E., Genetic transformation of potato (Solanum tuberosum): An efficient method to obtain transgenic plants, Plant Science, 59 (1988), 175-181

5 Umbarger, H.E., in Synthesis of Amino Acids and Proteins, (1975) pp1-56, MTP International Review of Science, Butterworth, London.

Wesley S.V., Helliwell C.A., Smith N.A., Wang M.B., Rouse D.T.,
10 Liu Q., Gooding P.S., Singh S.P., Abbott D., Stoutjesdijk P.A., Robinson S.P., Gleave A.P., Green A.G., Waterhouse P.M., Construct design for efficient, effective and high-throughput gene silencing in plants, Plant J 2001 Sep;27(6):581-90

15

20

25

30

35

40

45

25

Claims

1. A method for transformation of potato plants by transforming
    potato plant cells with an expression vector comprising

    a)   regulatory sequences of a promoter active in plants;

    b)   operably linked thereto a DNA sequence encoding a protein
         with the biological activity of an AHA synthase resistant
         to inhibitors of potato plant wildtype AHA synthase; and

    c)   operably linked thereto regulatory sequences which serve
         as transcription termination and/or polyadenylation
         signals in plants,

    selecting for AHA synthase inhibitor resistant cells and
    regenerating them to transgenic plants.

2. A method for transformation according to claim 1, wherein
    the expression vector comprises a DNA sequence according to
    SEQ-ID No. 1.

3. A method for transformation according to claim 1, wherein the
    DNA sequence encoding a protein with the biological activity
    of an AHA synthase resistant to inhibitors of potato plant
    wildtype AHA synthase is selected from the group consisting
    of

    a)   DNA sequence comprising a nucleotide according to
         SEQ-ID NO. 1;

    b)   DNA sequence comprising a nucleotide sequence which
         hybridizes to a complementary strand of the nucleotide
         sequence a)

    c)   DNA sequence comprising a nucleotide sequence which is
         degenerate to the nucleotide sequence of a) and

    d)   DNA sequence being a derivative, analogue or fragment
         of a nucleotide sequence of a), b) or c) and encoding a
         protein possessing AHA synthase activity and conferring
         resistance to AHA synthase inhibitors.

0093/387/2002 K/fr   08.07.2002   Zeichn. + Sequ.

26

4. A method for transformation according to any of claims 1
    to 3, wherein the AHA synthase promoter from Arabidopsis
    thaliana or the nos promoter is used.

5 5. A method for transformation according to any of claims 1
    to 4, wherein the AHA synthase terminator from Arabidopsis
    thaliana or the OCS terminator is used.

6. A method for transformation according to any of claims 1
10   to 5, wherein for selection a imidazolinone type herbicide
    is used.

7. A method for transformation according to claim 6, wherein for
    selection (RS)-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-
15   2-yl)-5-methoxymethylnicotinic acid is used.

8. A plant expression vector according to claim 1 or 2 addition-
    ally comprising a heterologous DNA sequence.

20 9. A plant expression vector according to claim 8, wherein
    the heterologous DNA sequence encodes a peptide, protein,
    antisense-, sense-RNA, viral RNA or ribozyme.

10. A plant expression vector according to claim 9, wherein the
25   heterologous DNA sequence contains information that causes
    changes in the carbohydrate concentration and the carbohy-
    drate composition of regenerated potato plants.

11. A plant expression vector according to claim 10, wherein the
30   heterologous DNA sequence contains information that causes
    the increased production of amylopectin type starches.

12. A plant expression vector according to claim 10, wherein the
    heterologous DNA sequence contains information that causes
35   the increased production of amylose type starches.

13. A transgenic potato plant cell produced by the method
    for transformation according to any of claims 1 to 7 and
    containing a plant expression vector according to any of
40   claims 8 to 12.

14. A transgenic potato plant produced by the method of trans-
    formation according to any of claims 1 to 7, wherein the
    regenerated plant exhibits an elevated resistance to
45   imidazolinone type herbicides.

15. Harvest product of the transgenic potato plant according
    to any of claims 13 and 14 comprising a DNA sequence
    SEQ ID No. 1 or a DNA sequence according to claim 3.

5 16. Harvest product according to claim 15 wherein the harvest
    product is a tuber.

17. Propagation material of transgenic potato plants comprising
    a DNA sequence SEQ ID No. 1 or a DNA sequence according to
10 claim 3.

18. Use of a DNA sequence SEQ ID No. 1 according to claim 2 or
    a DNA sequence according to claim 3, or a plant expression
    vector according to of any of claims 8 to 12 in potato plant
15 cells, potato tissue cultures, potato plants and/or potato
    plant breeding.

20

25

30

35

40

45

Abstract

The present invention relates to the use of mutated AHAS genes
5 conferring resistance to herbicides and resulting in an highly
efficient selection system for the production of transgenic
potato lines.

The invention provides a key advantage by minimizing the escapes
10 to almost zero of all shoots regenerated without interfering with
the high number of shoots regenerated per explant.

15

20

25

30

35

40

45

SEQUENCE LISTING

<110> BASF Plant Science GmbH

<120> Use of AHAS mutant genes as selection marker in potato
transformation

<130> NAE387-02

<140> 0093-53700
<141> 2002-07-08

<160> 2

<170> PatentIn Ver. 2.1

<210> 1
<211> 5717
<212> DNA
<213> Arabidopsis thaliana

<220>
<221> CDS
<222> (2484)..(4493)

<400> 1
tctagattat gtatttccaa ctttcattaa caatataatc gcatataaat gaaaaatcgt 60

ttccaggata atattttgat gaaatctcat attattgttc gtactcggat tgatgttgaa 120

ggcttgaagc gcttcaaatt atagaccaga ttatttaagt ttttcttttg tttactccat 180

atcaatttga tccattatac tacctaagaa aatttaggta acatagaatt atttattgtt 240

atagtaaaaa aaaggaaaac cacaaaaata atctactttt acgtatatac tattttcatg 300

acataagtaa ttaagttgta caactttttt ttaatgaaaa gagagagtaa atttatcatg 360

ttcatgtgta gttacctcgt gaataaccga cggttatata gacgcctaac atgaattgtt 420

cagttgaaga cagttcaaaa catgtgtttc actctaaaat cctcaacaaa aaaaagtgt 480

taaaatttgt aaacctcttt caagcaaaaa agaaaaagt gttagaatcc caagattctt 540

tcataatccg gaatcttggc tgaaaacgta taaagagat tgacgtagta acaggagtc 600

ttggtatgct tccatgcttt ttatcctttt ttgtcatgga accatgattt ggttaccatt 660

tattatgtaa ccgaaatttt cattgtaata atgaatattt aaattttag caaaaaaaa 720

caaaaaaaa caaggagtct tgtcttcgtt ctcaaatttc agagctcttg cacttttcaa 780

gagttttact ttgatgagtg agacatttgt ctttttagtg tttattttct aaacttaaaa 840

tagtagcatc aacatcactc aattataatt cttaagatgt tgtagaaaaa tatttttatag 900

atggaaagta atcgatatta agacaaataa gaaaccaaac cggactttgt gttcagaccg 960

2

aatcaaatct gaattggaga aattatggtg gaggcgaaag tcaacggaac taaagtataa 1020

aaccaaatgt caaaaataaa acccaatttt catccttaaa cgaacctgct gaaaccctaa 1080

tttcgattac caattccgat ctaaaaagaa gtcatggaag ccattgattc cgcaatcgat 1140

cctctcagag atttcgctaa gagcagtgtt cgtctcgtcc agcgctgtca caaacccgat 1200

cgcaagggta acgccttttc tcaaaaaaat ctcattccg attttgatc tgtagattag 1260

ggttttctga aattttgata tcatttgtaa ttgaattggt tatcagaatt cacgaaagta 1320

gctgtgcgta cggcgattgg atttgtggtg atgggattcg ttggattctt cgtgaagctc 1380

gttttcatcc caatcaacaa catcatcgtt ggatcttctt agtgtagtac tttctttacg 1440

aggtaattga tctcgcatta tatatctaca ttttggttat gttacttgac atatagtcat 1500

tgattcaata gttctgttaa ttcctttaaa gatcattttg actagaccac attcttggtt 1560

cattcctcaa taatttgtaa tcatattggt ggatatagaa gtagattggt tatagatcag 1620

atagtggaag actttaggat gaatttcagc tagttttttt ttttggctta ttgtctcaaa 1680

agattagtgc tttgctgtct ccattgcttc tgctatcgac acgcttctgt ctccttgtat 1740

ctttattata tctattcgtc ccatgagttt tgtttgttct gtattcgttc gctctggtgt 1800

catggatgga gtctctgttc catgtttctg taatgcatgt tgggttgttt catgcaagaa 1860

atgctgagat aaacactcat ttgtgaaagt ttctaaactc tgaatcgcgc tacaggcaat 1920

gctccgagga gtaggaggag aagaacgaac caaacgacat tatcagccct ttgaggaagc 1980

tcttagtttt gttattgttt ttgtagccaa attctccatt cttattccat tttcacttat 2040

ctcttgttcc ttatagacct tataagtttt ttattcatgt atacaaatta tattgtcatc 2100

aagaagtatc tttaaaatct aaatctcaaa tcaccaggac tatgtttttg tccaattcgt 2160

ggaaccaact tgcagcttgt atccattctc ttaaccaata aaaaagaaa gaaagatcaa 2220

tttgataaat ttctcagcca caaattctac atttaggttt tagcatatcg aaggctcaat 2280

cacaaataca atagatagac tagagattcc agcgtcacgt gagtttcatc tataaataaa 2340

ggaccaaaaa tcaaatcccg agggcatttt cgtaatccaa cataaaaccc ttaaacttca 2400

agtctcattt ttaaacaaat catgttcaca agtctcttct tcttctctgt ttctctatct 2460

cttgctcatc tttctcctga acc atg gcg gcg gca aca aca aca aca aca aca 2513
                         Met Ala Ala Ala Thr Thr Thr Thr Thr Thr
                          1   5   10

tct tct tcg atc tcc ttc tcc acc aaa cca tct cct tcc tcc tcc aaa   2561
Ser Ser Ser Ile Ser Phe Ser Thr Lys Pro Ser Pro Ser Ser Ser Lys

```
                 15                          20                          25

tca cca tta cca atc tcc aga ttc tcc ctc cca ttc tcc cta aac ccc     2609
Ser Pro Leu Pro Ile Ser Arg Phe Ser Leu Pro Phe Ser Leu Asn Pro
            30                  35                  40

aac aaa tca tcc tcc tcc tcc cgc cgc cgc ggt atc aaa tcc agc tct     2657
Asn Lys Ser Ser Ser Ser Ser Arg Arg Arg Gly Ile Lys Ser Ser Ser
            45                  50                  55

ccc tcc tcc atc tcc gcc gtg ctc aac aca acc acc aat gtc aca acc     2705
Pro Ser Ser Ile Ser Ala Val Leu Asn Thr Thr Thr Asn Val Thr Thr
            60                  65                  70

act ccc tct cca acc aaa cct acc aaa ccc gaa aca ttc atc tcc cga     2753
Thr Pro Ser Pro Thr Lys Pro Thr Lys Pro Glu Thr Phe Ile Ser Arg
75                  80                  85                  90

ttc gct cca gat caa ccc cgc aaa ggc gct gat atc ctc gtc gaa gct     2801
Phe Ala Pro Asp Gln Pro Arg Lys Gly Ala Asp Ile Leu Val Glu Ala
                    95                  100                 105

tta gaa cgt caa ggc gta gaa acc gta ttc gct tac cct gga ggt gca     2849
Leu Glu Arg Gln Gly Val Glu Thr Val Phe Ala Tyr Pro Gly Gly Ala
            110                 115                 120

tca atg gag att cac caa gcc tta acc cgc tct tcc tca atc cgt aac     2897
Ser Met Glu Ile His Gln Ala Leu Thr Arg Ser Ser Ser Ile Arg Asn
            125                 130                 ·135

gtc ctt cct cgt cac gaa caa gga ggt gta ttc gca gca gaa gga tac     2945
Val Leu Pro Arg His Glu Gln Gly Gly Val Phe Ala Ala Glu Gly Tyr
            140                 145                 150

gct cga tcc tca ggt aaa cca ggt atc tgt ata gcc act tca ggt ccc     2993
Ala Arg Ser Ser Gly Lys Pro Gly Ile Cys Ile Ala Thr Ser Gly Pro
155                 160                 165                 170

gga gct aca aat ctc gtt agc gga tta gcc gat gcg ttg tta gat agt     3041
Gly Ala Thr Asn Leu Val Ser Gly Leu Ala Asp Ala Leu Leu Asp Ser
            175                 180                 185

gtt cct ctt gta gca atc aca gga caa gtc cct cgt cgt atg att ggt     3089
Val Pro Leu Val Ala Ile Thr Gly Gln Val Pro Arg Arg Met Ile Gly
            190                 195                 200

aca gat gcg ttt caa gag act ccg att gtt gag gta acg cgt tcg att     3137
Thr Asp Ala Phe Gln Glu Thr Pro Ile Val Glu Val Thr Arg Ser Ile
            205                 210                 ·215

acg aag cat aac tat ctt gtg atg gat gtt gaa gat atc cct agg att     3185
Thr Lys His Asn Tyr Leu Val Met Asp Val Glu Asp Ile Pro Arg Ile
            220                 225                 230

att gag gaa gct ttc ttt tta gct act tct ggt aga cct gga cct gtt     3233
Ile Glu Glu Ala Phe Phe Leu Ala Thr Ser Gly Arg Pro Gly Pro Val
235                 240                 245                 250
```

```
ttg gtt gat gtt cct aaa gat att caa caa cag ctt gcg att cct aat    3281
Leu Val Asp Val Pro Lys Asp Ile Gln Gln Gln Leu Ala Ile Pro Asn
            255             260                 265

tgg gaa cag gct atg aga tta cct ggt tat atg tct agg atg cct aaa    3329
Trp Glu Gln Ala Met Arg Leu Pro Gly Tyr Met Ser Arg Met Pro Lys
            270             275                 280

cct ccg gaa gat tct cat ttg gag cag att gtt agg ttg att tct gag    3377
Pro Pro Glu Asp Ser His Leu Glu Gln Ile Val Arg Leu Ile Ser Glu
            285             290                 295

tct aag aag cct gtg ttg tat gtt ggt ggt ggt tgt ttg aat tct agc    3425
Ser Lys Lys Pro Val Leu Tyr Val Gly Gly Gly Cys Leu Asn Ser Ser
            300             305                 310

gat gaa ttg ggt agg ttt gtt gag ctt acg ggg atc cct gtt gcg agt    3473
Asp Glu Leu Gly Arg Phe Val Glu Leu Thr Gly Ile Pro Val Ala Ser
315             320             325                 330

acg ttg atg ggg ctg gga tct tat cct tgt gat gat gag ttg tcg tta    3521
Thr Leu Met Gly Leu Gly Ser Tyr Pro Cys Asp Asp Glu Leu Ser Leu
            335             340                 345

cat atg ctt gga atg cat ggg act gtg tat gca aat tac gct gtg gag    3569
His Met Leu Gly Met His Gly Thr Val Tyr Ala Asn Tyr Ala Val Glu
            350             355                 360

cat agt gat ttg ttg ttg gcg ttt ggg gta agg ttt gat gat cgt gtc    3617
His Ser Asp Leu Leu Leu Ala Phe Gly Val Arg Phe Asp Asp Arg Val
            365             370                 375

acg ggt aag ctt gag gct ttt gct agt agg gct aag att gtt cat att    3665
Thr Gly Lys Leu Glu Ala Phe Ala Ser Arg Ala Lys Ile Val His Ile
            380             385                 390

gat att gac tcg gct gag att ggg aag aat aag act cct cat gtg tct    3713
Asp Ile Asp Ser Ala Glu Ile Gly Lys Asn Lys Thr Pro His Val Ser
395             400             405                 410

gtg tgt ggt gat gtt aag ctg gct ttg caa ggg atg aat aag gtt ctt    3761
Val Cys Gly Asp Val Lys Leu Ala Leu Gln Gly Met Asn Lys Val Leu
            415             420                 425

gag aac cga gcg gag gag ctt aag ctt gat ttt gga gtt tgg agg aat    3809
Glu Asn Arg Ala Glu Glu Leu Lys Leu Asp Phe Gly Val Trp Arg Asn
            430             435                 440

gag ttg aac gta cag aaa cag aag ttt ccg ttg agc ttt aag acg ttt    3857
Glu Leu Asn Val Gln Lys Gln Lys Phe Pro Leu Ser Phe Lys Thr Phe
            445             450                 455

ggg gaa gct att cct cca cag tat gcg att aag gtc ctt gat gag ttg    3905
Gly Glu Ala Ile Pro Pro Gln Tyr Ala Ile Lys Val Leu Asp Glu Leu
            460             465                 470
```

```
act gat gga aaa gcc ata ata agt act ggt gtc ggg caa cat caa atg   3953
Thr Asp Gly Lys Ala Ile Ile Ser Thr Gly Val Gly Gln His Gln Met
475             480             485             490

tgg gcg gcg cag ttc tac aat tac aag aaa cca agg cag tgg cta tca   4001
Trp Ala Ala Gln Phe Tyr Asn Tyr Lys Lys Pro Arg Gln Trp Leu Ser
            495             500             505

tca gga ggc ctt gga gct atg gga ttt gga ctt cct gct gcg att gga   4049
Ser Gly Gly Leu Gly Ala Met Gly Phe Gly Leu Pro Ala Ala Ile Gly
        510             515             520

gcg tct gtt gct aac cct gat gcg ata gtt gtg gat att gac gga gat   4097
Ala Ser Val Ala Asn Pro Asp Ala Ile Val Val Asp Ile Asp Gly Asp
        525             530             535

gga agc ttt ata atg aat gtg caa gag cta gcc act att cgt gta gag   4145
Gly Ser Phe Ile Met Asn Val Gln Glu Leu Ala Thr Ile Arg Val Glu
    540             545             550

aat ctt cca gtg aag gta ctt tta tta aac aac cag cat ctt ggc atg   4193
Asn Leu Pro Val Lys Val Leu Leu Leu Asn Asn Gln His Leu Gly Met
555             560             565             570

gtt atg caa tgg gaa gat cgg ttc tac aaa gct aac cga gct cac aca   4241
Val Met Gln Trp Glu Asp Arg Phe Tyr Lys Ala Asn Arg Ala His Thr
            575             580             585

ttt ctc ggg gat ccg gct cag gag gac gag ata ttc ccg aac atg ttg   4289
Phe Leu Gly Asp Pro Ala Gln Glu Asp Glu Ile Phe Pro Asn Met Leu
            590             595             600

ctg ttt gca gca gct tgc ggg att cca gcg gcg agg gtg aca aag aaa   4337
Leu Phe Ala Ala Ala Cys Gly Ile Pro Ala Ala Arg Val Thr Lys Lys
        605             610             615

gca gat ctc cga gaa gct att cag aca atg ctg gat aca cca gga cct   4385
Ala Asp Leu Arg Glu Ala Ile Gln Thr Met Leu Asp Thr Pro Gly Pro
    620             625             630

tac ctg ttg gat gtg att tgt ccg cac caa gaa cat gtg ttg ccg atg   4433
Tyr Leu Leu Asp Val Ile Cys Pro His Gln Glu His Val Leu Pro Met
635             640             645             650

atc ccg aat ggt ggc act ttc aac gat gtc ata acg gaa gga gat ggc   4481
Ile Pro Asn Gly Gly Thr Phe Asn Asp Val Ile Thr Glu Gly Asp Gly
            655             660             665

cgg att aaa tac tgagagatga aaccggtgat tatcagaacc ttttatggtc   4533
Arg Ile Lys Tyr
        670


tttgtatgca tatggtaaaa aaacttagtt tgcaatttcc tgtttgtttt ggtaatttga 4593

gtttctttta gttgttgatc tgcctgcttt ttggtttacg tcagactact actgctgttg 4653

ttgtttggtt tcctttcttt cattttataa ataaataatc cggttcggtt tactccttgt 4713
```

```
gactggctca gtttggttat tgcgaaatgc gaatggtaaa ttgagtaatt gaaattcgtt 4773

attagggttc taagctgttt taacagtcac tgggttaata tctctcgaat cttgcatgga 4833

aaatgctctt accattggtt tttaattgaa atgtgctcat atgggccgtg gtttccaaat 4893

taaataaaac tacgatgtca tcgagaagta aaatcaactg tgtccacatt atcagttttg 4953

tgtatacgat gaaatagggt aattcaaaat ctagcttgat atgccttttg gttcatttta 5013

accttctgta aacatttttt cagattttga acaagtaaat ccaaaaaaaa aaaaaaaaaa 5073

tctcaactca acactaaatt attttaatgt ataaaagatg cttaaaacat ttggcttaaa 5133

agaagaagc taaaaacata gagaactctt gtaaattgaa gtatgaaaat atactgaatt 5193

gggtattata tgaatttttc tgatttagga ttcacatgat ccaaaaagga aatccagaag 5253

cactaatcag acattggaag taggaatatt tcaaaaagtt ttttttttt aagtaagtga 5313

caaaagcttt taaaaaatag aaaagaaact agtattaaag ttgtaaattt aataaacaaa 5373

agaaattttt tatatttttt catttctttt tccagcatga ggttatgatg gcaggatgtg 5433

gatttcattt ttttcctttt gatagccttt taattgatct attataattg acgaaaaaat 5493

attagttaat tatagatata ttttaggtag tattagcaat ttacacttcc aaaagactat 5553

gtaagttgta aatatgatgc gttgatctct tcatcattca atggttagtc aaaaaaataa 5613

aagcttaact agtaaactaa agtagtcaaa aattgtactt tagtttaaaa tattacatga 5673

ataatccaaa acgacattta tgtgaaacaa aaacaatatc taga                  5717
```

```
<210> 2
<211> 670
<212> PRT
<213> Arabidopsis thaliana

<400> 2
Met Ala Ala Ala Thr Thr Thr Thr Thr Thr Ser Ser Ser Ile Ser Phe
1               5                   10                  15

Ser Thr Lys Pro Ser Pro Ser Ser Ser Lys Ser Pro Leu Pro Ile Ser
            20                  25                  30

Arg Phe Ser Leu Pro Phe Ser Leu Asn Pro Asn Lys Ser Ser Ser Ser
        35                  40                  45

Ser Arg Arg Arg Gly Ile Lys Ser Ser Ser Pro Ser Ser Ile Ser Ala
    50                  55                  60

Val Leu Asn Thr Thr Thr Asn Val Thr Thr Thr Pro Ser Pro Thr Lys
65                  70                  75                  80
```

Pro Thr Lys Pro Glu Thr Phe Ile Ser Arg Phe Ala Pro Asp Gln Pro
                85   90   95

Arg Lys Gly Ala Asp Ile Leu Val Glu Ala Leu Glu Arg Gln Gly Val
        100   105   110

Glu Thr Val Phe Ala Tyr Pro Gly Gly Ala Ser Met Glu Ile His Gln
        115   120   125

Ala Leu Thr Arg Ser Ser Ser Ile Arg Asn Val Leu Pro Arg His Glu
        130   135   140

Gln Gly Gly Val Phe Ala Ala Glu Gly Tyr Ala Arg Ser Ser Gly Lys
145   150   155   160

Pro Gly Ile Cys Ile Ala Thr Ser Gly Pro Gly Ala Thr Asn Leu Val
                165   170   175

Ser Gly Leu Ala Asp Ala Leu Leu Asp Ser Val Pro Leu Val Ala Ile
        180   185   190

Thr Gly Gln Val Pro Arg Arg Met Ile Gly Thr Asp Ala Phe Gln Glu
        195   200   205

Thr Pro Ile Val Glu Val Thr Arg Ser Ile Thr Lys His Asn Tyr Leu
210   215   220

Val Met Asp Val Glu Asp Ile Pro Arg Ile Ile Glu Glu Ala Phe Phe
225   230   235   240

Leu Ala Thr Ser Gly Arg Pro Gly Pro Val Leu Val Asp Val Pro Lys
                245   250   255

Asp Ile Gln Gln Gln Leu Ala Ile Pro Asn Trp Glu Gln Ala Met Arg
                260   265   270

Leu Pro Gly Tyr Met Ser Arg Met Pro Lys Pro Pro Glu Asp Ser His
        275   280   285

Leu Glu Gln Ile Val Arg Leu Ile Ser Glu Ser Lys Lys Pro Val Leu
        290   295   300

Tyr Val Gly Gly Gly Cys Leu Asn Ser Ser Asp Glu Leu Gly Arg Phe
305   310   315   320

Val Glu Leu Thr Gly Ile Pro Val Ala Ser Thr Leu Met Gly Leu Gly
                325   330   335

Ser Tyr Pro Cys Asp Asp Glu Leu Ser Leu His Met Leu Gly Met His
                340   345   350

Gly Thr Val Tyr Ala Asn Tyr Ala Val Glu His Ser Asp Leu Leu Leu
        355   360   365

Ala Phe Gly Val Arg Phe Asp Asp Arg Val Thr Gly Lys Leu Glu Ala
        370   375   380

Phe Ala Ser Arg Ala Lys Ile Val His Ile Asp Ile Asp Ser Ala Glu
385   390   395   400

Ile Gly Lys Asn Lys Thr Pro His Val Ser Val Cys Gly Asp Val Lys
                405   410   415

Leu Ala Leu Gln Gly Met Asn Lys Val Leu Glu Asn Arg Ala Glu Glu
            420   425   430

Leu Lys Leu Asp Phe Gly Val Trp Arg Asn Glu Leu Asn Val Gln Lys
            435   440   445

Gln Lys Phe Pro Leu Ser Phe Lys Thr Phe Gly Glu Ala Ile Pro Pro
450   455   460

Gln Tyr Ala Ile Lys Val Leu Asp Glu Leu Thr Asp Gly Lys Ala Ile
465   470   475   480

Ile Ser Thr Gly Val Gly Gln His Gln Met Trp Ala Ala Gln Phe Tyr
            485   490   495

Asn Tyr Lys Lys Pro Arg Gln Trp Leu Ser Ser Gly Gly Leu Gly Ala
            500   505   510

Met Gly Phe Gly Leu Pro Ala Ala Ile Gly Ala Ser Val Ala Asn Pro
    515   520   525

Asp Ala Ile Val Val Asp Ile Asp Gly Asp Gly Ser Phe Ile Met Asn
530   535   540

Val Gln Glu Leu Ala Thr Ile Arg Val Glu Asn Leu Pro Val Lys Val
545   550   555   560

Leu Leu Leu Asn Asn Gln His Leu Gly Met Val Met Gln Trp Glu Asp
            565   570   575

Arg Phe Tyr Lys Ala Asn Arg Ala His Thr Phe Leu Gly Asp Pro Ala
            580   585   590

Gln Glu Asp Glu Ile Phe Pro Asn Met Leu Leu Phe Ala Ala Ala Cys
    595   600   605

Gly Ile Pro Ala Ala Arg Val Thr Lys Lys Ala Asp Leu Arg Glu Ala
    610   615   620

Ile Gln Thr Met Leu Asp Thr Pro Gly Pro Tyr Leu Leu Asp Val Ile
625   630   635   640

Cys Pro His Gln Glu His Val Leu Pro Met Ile Pro Asn Gly Gly Thr
            645   650   655

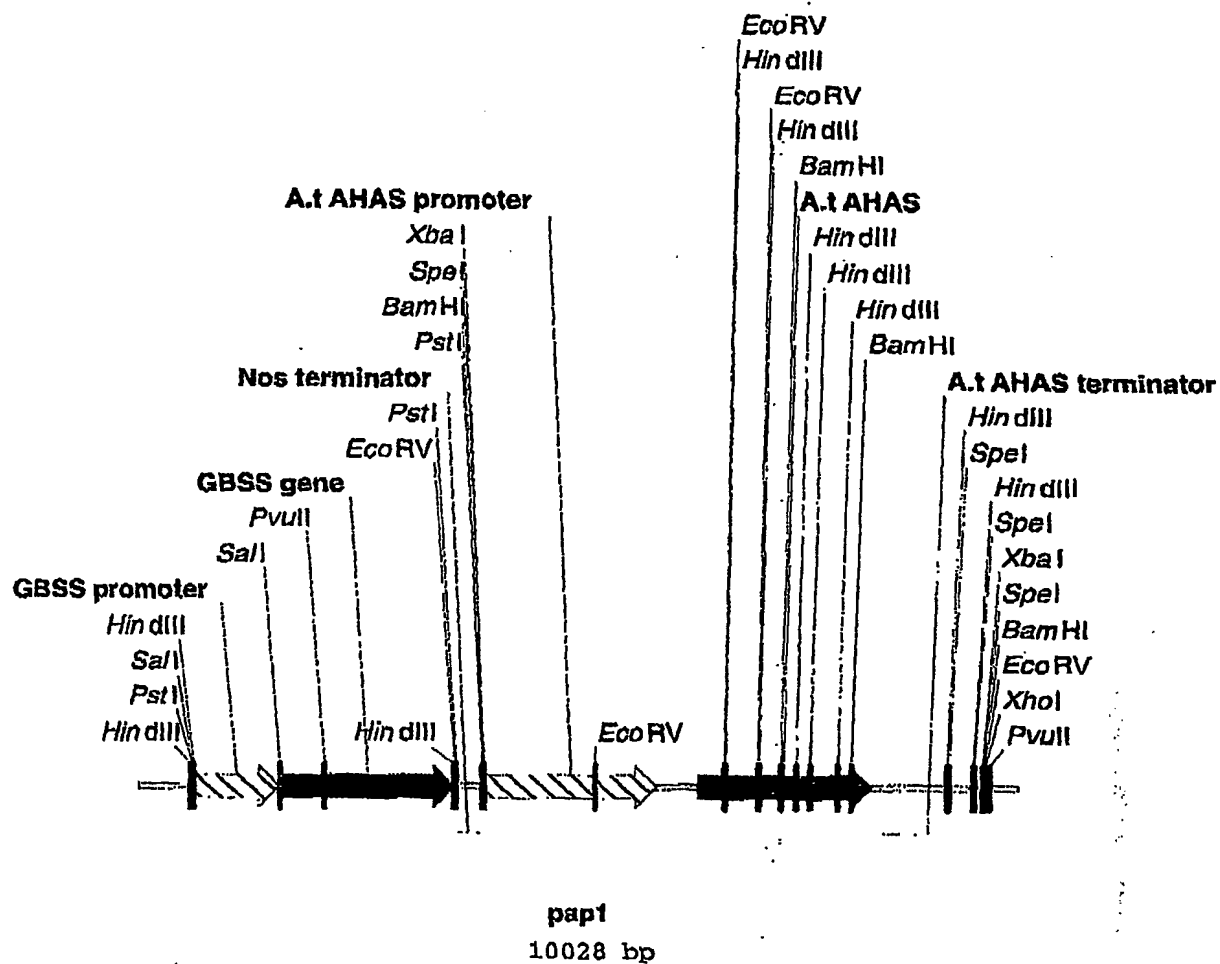Phe Asn Asp Val Ile Thr Glu Gly Asp Gly Arg Ile Lys Tyr
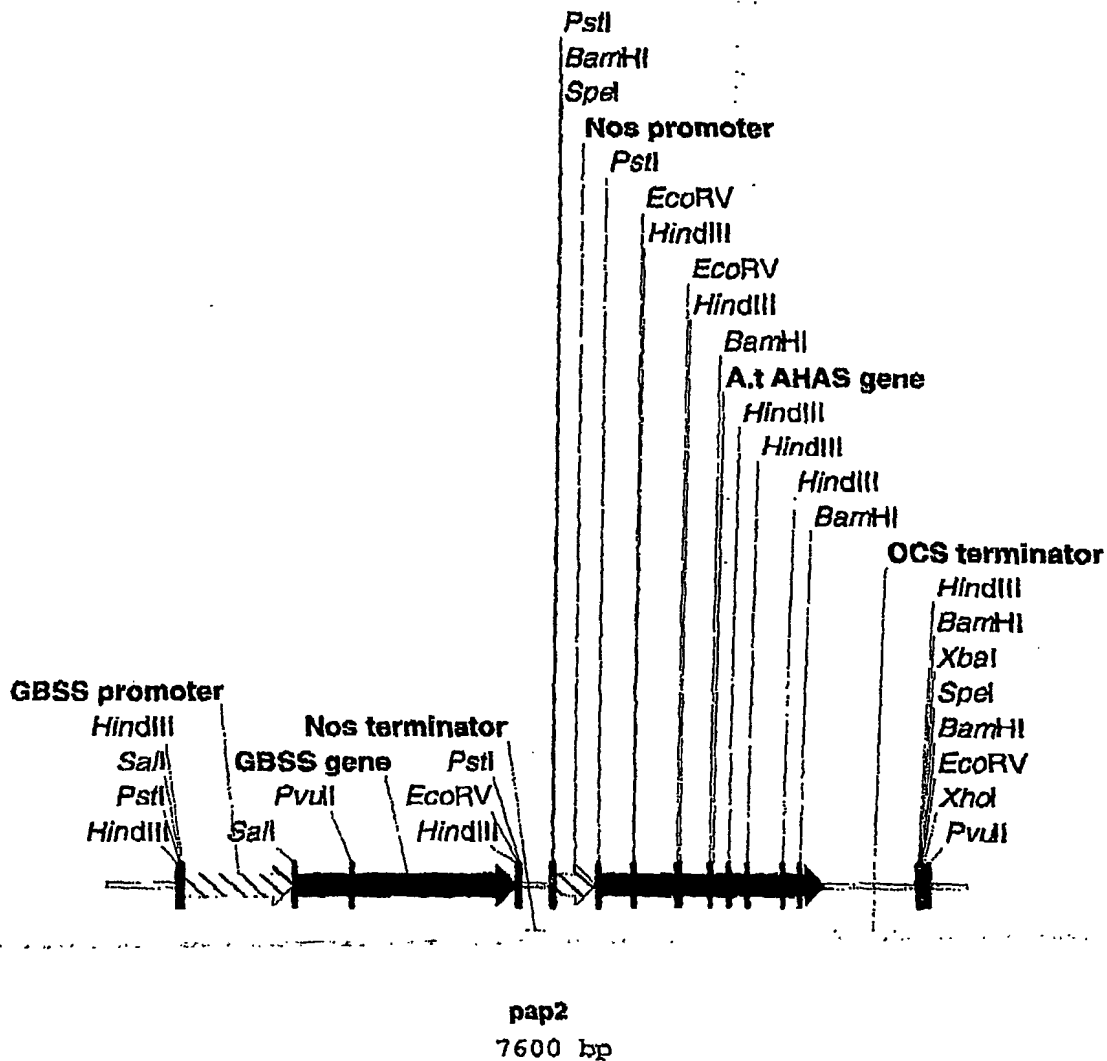   660   665   670

Figure 1. T–DNA of pAP1 construct

Pstl
BamHI
Spel
Nos promoter
Pstl
EcoRV
HindIII
EcoRV
HindIII
BamHI
A.t AHAS gene
HindIII
HindIII
HindIII
BamHI
OCS terminator
HindIII
BamHI
Xbal
Spel
BamHI
EcoRV
Xhol
Pvul

GBSS promoter
HindIII
Sall
Pstl
HindIII
Sall
GBSS gene
Pvul
Nos terminator
Pstl
EcoRV
HindIII

pap2
7600 bp

Figure 2. T–DNA of pAP2 construct